III. FAT-DEFICIENCY DISEASE OF RATS. THE RELATION OF THE ESSENTIAL UNSATURATED ACIDS TO TUMOUR FORMATION IN THE ALBINO RAT ON NORMAL DIET

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Rats which have been fed from the time of weaning on a diet completely free from fat gradually cease to gain in weight, and after being thus dieted for from 4 to 6 months their weight remains constant. This condition is accompanied by other symptoms, of which a dry scurfy skin and the so-called scaly tail are the most obvious [Burr & Burr, 1930]. The animals can be cured by giving them small doses of linoleic or arachidonic acids, the latter being the most potent in promoting growth. The fat-starved rat which is receiving an unlimited diet of carbohydrate, protein and the necessary accessory factors, is still able to make and store fat; indeed, the proportion of fat in rats after a very long period of fat-starvation was found to be exceptionally high [Smedley-MacLean & Nunn, 1940; Hume *et al.* 1940]. When daily doses of 7–14 mg. methyl arachidonate were given to rats which had been for many months on a fat-free diet, growth was resumed and analysis of the carcass 5 weeks later showed that the increased weight was accompanied by a diminution of the proportion of fat present when compared with animals which were still receiving only the fat-free diet. It was also observed that only a fraction of the arachidonic acid which had been administered was found in the body [Smedley-MacLean & Nunn, 1940].

The part played by the highly unsaturated acid does not appear therefore to be concerned with the formation of fat from carbohydrate or with its storage. It does, however, play some essential role in bringing about an increase in weight which is not due to the deposition of fat and which probably therefore involves the formation of new cells. The diminished store of fat found in the carcass and subcutaneous tissue when curative doses of unsaturated acids were given suggested the possibility that, in the process of growth, this diminution of fat might be directly connected with the formation of new tissue, and that the process might possibly be in some way linked up with the part played by these essential unsaturated acids.

We desired therefore to investigate the changes in the content both of fat and of highly unsaturated acid in some other condition where the formation of new cell tissue was actively proceeding. Such a condition is provided by the rapidly growing Walker tumour grafted under the skin of the rat. We therefore enlisted the co-operation of Dr Haddow to whom we are greatly indebted for providing us with the necessary material.

The rats selected were 100–150 g. in weight and were fed throughout the experiment on a diet rich in fat; it contained milk, oil cake and fish-meal so that there was a good supply of unsaturated acids. Generally speaking, in 10–14 days

after the tumour tissue had been implanted, large tumours had developed. Rats of the same strain as those into which the tumours were to be implanted and of approximately the same weight, were taken as controls and were killed at the same time that the tumours were implanted in the experimental animals. The amounts of fat and of polyunsaturated acids were estimated in the skin, liver and carcass of the controls. In some experiments a second group of controls was killed about a fortnight later at the same time as the rats bearing the tumours. The rats were not litter-mates and they varied considerably in the proportion of fat they contained.

Estimation of fat. The tumour was dissected out from its bed of fat, the liver removed, the skin with the subcutaneous tissue detached and the remainder of the carcass, exclusive of tail, head, paws and alimentary canal, minced. These fractions, tumour, liver, skin and carcass were dehydrated by immersion in acetone for at least 48 hr.; the extracts were filtered and the residues boiled with 96% alcohol for 2 hr. in an atmosphere of nitrogen. After filtering, the residual tissue was extracted with ether in a Soxhlet apparatus for 2 days; the acetone and alcoholic extracts were taken to dryness and the ether-soluble part of the dissolved matter extracted. The ethereal extracts were added together, dried with anhydrous Na_2SO_4, the ether distilled off and the total lipoid matter taken to constant weight *in vacuo*. As far as possible these operations were carried out in an atmosphere of nitrogen.

Estimation of polyunsaturated acids. The method of estimation of these acids depends on the fact that when bromine is added to their solution in ether or benzene, a mixture of isomeric bromides is formed of which only a small proportion is insoluble in ether and a still smaller proportion in benzene. These proportions however, though small, are constant.

The method of estimation adopted in our earlier experiments was as follows. A weighed amount of fat was saponified by keeping it overnight with excess of 10% alcoholic KOH; the alcohol was then distilled off, the soap dissolved in water, acidified with H_2SO_4 and the acids extracted with ether. The residue from the ethereal solution was dissolved in benzene, the solution dried with anhydrous Na_2SO_4, concentrated, ice-cooled and carefully treated with bromine until a slight excess was present. No preliminary separation into saturated and unsaturated acids was made. After 48 hr. in the cold room, the precipitate was filtered off, repeatedly washed with ether and weighed.

This method was tested by adding known quantities of arachidonic acid obtained from beef suprarenal glands to a mixture of oleic and palmitic acids in benzene solution. It gave comparative results which, though a high degree of accuracy cannot be claimed, furnish a satisfactory basis of comparison where small differences are not regarded as significant. The addition of small quantities of cholesterol did not influence the precipitation. The difficulties in the estimation of arachidonic acid have been discussed elsewhere [Dolby *et al.* 1940]. Since linoleic acid tetrabromide and linolenic acid hexabromide are soluble in benzene they are not precipitated when the precipitation is carried out in benzene solution; under this condition only the benzene-insoluble bromides of arachidonic ($C_{20}H_{32}O_2$) and clupanodonic ($C_{22}H_{34}O_2$) acids, the two acids especially characteristic of animal fat, will be precipitated. Bromine estimations made on the precipitates in these experiments gave generally between 65 and 70% Br, showing that the precipitates consisted mainly of mixtures of the octabromide of arachidonic acid and the decabromide of clupanodonic acid.

The bromide obtained from fatty acids of the livers of group IV of the tumour-bearing animals was separated by repeated extraction with hot benzene

into two fractions which were then completely analysed in Dr Weiler's laboratory. The results were as follows:

	% C	% H	% Br
Benzene-insoluble fraction	25·38	3·27	67·8
Theory for $C_{20}H_{32}O_2Br_8$	25·42	3·39	67·7
Benzene-soluble fraction	26·50	3·86	63·2
Theory for $C_{22}H_{36}O_4Br_8$	26·28	3·58	63·74

The variations in bromine estimations obtained may perhaps be partly due to the presence of polybromo derivatives of partially oxidized acids. In the experiments now described derivatives of arachidonic and clupanodonic acids were certainly present, but as the rats had been fed with a mixture containing fish-meal and oil cake, it is possible that the diet had included partially oxidized acids.

The isolation of a dihydroarachidonic acid hexabromide from the tissues of the fat-starved rat has been previously described [Smedley-MacLean & Nunn, 1940].

The method of precipitation in benzene solution has the disadvantage that the solid isomer of arachidonic bromide insoluble in benzene is only a very small proportion of the total octabromide formed and the *total* weight of bromide precipitated must be multiplied by 2·9 to arrive at the amount of arachidonic acid present. In our final experiment we precipitated the bromides in ether solution; here, the amount of ether-insoluble bromide multiplied by the factor 1·3 was taken as the weight of arachidonic present. In this case if any linolenic acid were present, which is unlikely, the bromide precipitate would also contain linolenic acid hexabromide. In any one experiment the same method of precipitation was always used for the controls and the experimental animals. The results are set forth in Table 1.

The proportion of lipoid material in the tissues of the tumour-bearing rat

The tumours lie embedded in a thick layer of subcutaneous fat. Estimations of the proportion of the total lipoid matter extracted by ether to the fat-free dry weight left after ether extraction in the various tissues, differed greatly in the control animals and even more widely in the tumour-bearing animals. Thus, in the control normal rats the percentage of lipoid to dry weight in the skin varied from 53·4 to 90·5, whilst in the tumour-bearing animals the variation was from 31 to 130. When the individual variations were so large it was obviously impossible to make any deduction unless a very large number of animals had been examined. In the carcass, the proportion of lipoid to fat-free dry weight varied from 32·6 to 41·6% in the controls and from 28·6 to 49·2% in the experimental animals. The corresponding figures for the liver were 11·5–21·1% in the controls and from 16·7 to 24·7% in the experimental animals.

The rats were fed both before and throughout the experiment on a diet rich in fat and the longest period they were allowed to survive after implantation of the tumour was 14 days. Under these conditions there was no evidence which could be regarded as significant of any change in the proportion of fat present, but as long as there was a plentiful supply both of fat and of unsaturated acid and absorption of fat from the intestine was taking place, even if increased utilization of fat occurred, it might not be apparent.

Changes in the proportion of the polyunsaturated acids (arachidonic and clupanodonic) during tumour growth

When the figures giving the proportion of highly unsaturated acid present in the skin were examined, there was a marked difference between those for the controls and those for the tumour-bearing animals.

Table 1. *Showing changes in the ratios of (1) weight of total lipoid to fat-free dry weight and (2) weight of arachidonic acid to fat-free dry weight, in the tissues of normal albino rats after the implantation of Walker tumour tissue*

	(a) Skin		(b) Carcass		(c) Liver	
	% lipoid dry wt.	% arachidonic acid dry wt.	% lipoid dry wt.	% arachidonic acid dry wt.	% lipoid dry wt.	% arachidonic acid dry wt.
Exp. 1:	Normal rats as controls					
4 rats killed, 9. x. 39	53·4	1·07	32·6	1·57	20·0	5·92
4 rats killed, 9. x. 39	60·6	1·19	36·2	1·95	14·7	3·86
Exp. 2:						
3 rats killed, 15. xi. 39	71·2	1·57	38·7	1·65	21·1	5·04
3 rats killed, 29. xi. 39	64·4	1·28	34·8	1·79	17·1	1·62
Exp. 4:						
3 rats killed, 29. iii. 40	79·8	1·57*	33·1	1·97*	16·9	1·83*
2 rats killed, 11. iv. 40	90·5	1·51*	41·6	1·62*	11·5	—
Mean value (calculated for 1 rat)	67·5	1·33	35·7	1·77	16·8	3·22
Exp. 1:	Rats with Walker tumours					
2 rats killed, 17. x. 39	59·7	0·93	37·2	1·42	19·6	2·53
3 rats killed, 17. x. 39 Tumours implanted, 9 x.	31·0	0·58	28·6	1·48	16·7	1·77
Exp. 2:						
5 rats killed, 29. xi. 39 Tumours implanted, 15. x. 39	44·8	0·81	31·1	1·77	24·4	5·16
Exp. 4:						
2 rats killed, 11. iv. 40	85·0	1·06*	45·0	1·62*	21·1	1·09*
2 rats killed, 11. iv. 40 Tumours implanted	130·0	0·87*	49·2	1·82*	24·7	3·41*
Mean calculated for 1 rat	61·9	0·82	36·0	1·64	21·6	3·22
Exp. 3:						
Controls for 3 rats in which tumours regressed or failed to grow	92·6	0·31	45·3	0·61	16·8	4·99

For the purpose of calculation, the unsaturated acid was regarded as arachidonic acid and its weight calculated as the total weight of bromide precipitated in benzene solution and washed with ether multiplied by 2·9 or as the total weight of insoluble bromide precipitated in ether solution (marked *), washed with ether and multiplied by 1·3.

We examined 22 control rats and 14 in which tumours were implanted. When the proportion of polyunsaturated acid to the weight of dry fat-free tissue was calculated, marked differences presented themselves (Table 1). In the skin of the tumour-bearing animals this proportion was reduced to less than two-thirds of that in the controls. In the 5 groups of controls, the values exceeded 1%, the average value being 1·33%. In 4 groups of tumour-bearing animals, the values were below 1% and in the 5th group 1·06%, the average being 0·82% and the limits of the values for the control and experimental animals did not overlap. The utilization of the essential unsaturated acid during the growth of the tumour was sufficient to reduce markedly the ratio of this acid to the dry weight of skin tissue even though the rat was supplied throughout the period of tumour growth with unlimited fat and unsaturated acids.

No such difference appeared in the carcass fat, and in the liver the results varied too widely for any conclusions to be drawn.

Table 2. *Showing weights recorded of (a) wet tissue, (b) dry tissue after ether-extraction, (c) total lipoid and (d) insoluble bromides of unsaturated acids, from normal albino rats and from those in which tumours had been implanted. The bromides were precipitated in benzene and washed with ether and their weight × 2·9 represents the weight of polyunsaturated acid calculated as arachidonic. The bromides (marked *) were precipitated in ether solution and their weight × 1·3 gives the weight of arachidonic acid. The 'dry wt.' is the weight of tissue left after extraction with alcohol and ether; the weight of ether-insoluble substance extracted by the alcohol being small. 'Lipoid' is the ether-soluble fraction consisting of fat, phospholipin and unsaponifiable matter*

			Normal albino rats															
			Carcass				Skin				Liver				Tumour			
Exp.	No. of rats	Wt. of rats g.	(a) Wet wt. g.	(b) Dry wt. g.	(c) Lipoid wt. g.	(d) Insoluble bromide wt. mg.	(a) Wet wt. g.	(b) Dry wt. g.	(c) Lipoid wt. g.	(d) Insoluble bromide wt. mg.	(a) Wet wt. g.	(b) Dry wt. g.	(c) Lipoid wt. g.	(d) Insoluble bromide wt. mg.	(a) Wet wt. g.	(b) Dry wt. g.	(c) Lipoid wt. g.	(d) Insoluble bromide wt. mg.
I	4	711·5	319·5	73·3	23·9	476	94·4	30·5	16·3	113	25·8	6·5	1·3	132	—	—	—	—
	4	502·9	261·0	59·5	21·6	431	76·3	24·7	14·9	100	24·2	5·9	0·9	79	—	—	—	—
II	3	296·9	149·6	35·8	13·8	222	51·6	15·7	11·2	85	11·6	2·8	0·6	49	—	—	—	—
	3	419·6	220·4	51·3	17·8	344	66·9	20·3	13·1	89	21·0	4·7	0·8	27	—	—	—	—
III	3	327·0	154·3	32·8	14·9	69	49·3	13·2	12·2	41	22·4	5·0	0·8	81	—	—	—	—
IV	3	326·0	155·5	33·4	11·1	508*	49·3	14·2	11·3	171*	23·1	5·7	1·0	36*	—	—	—	—
	2	280·6	131·0	27·5	11·4	342*	32·6	10·3	>9·3	119*	20·3	4·4	0·5	—*	—	—	—	—
			Rats with Walker tumours															
I	2	220	96·6	22·1	8·3	108	35·8	10·5	6·3	34	12·3	2·7	0·5	24	7·2	1·5	0·7	11
	3	350	147·2	33·1	9·5	168	44·2	15·2	5·2	30	26·6	5·9	1·0	36	33·5	3·9	0·7	19
II	5	762	326·9	73·3	22·5	449	128·6	34·6	15·5	96	45·7	9·4	2·3	168	69·6	8·1	1·3	70
III	Tumours failed to develop in experimental animals																	
IV	2	274	109·5	21·8	9·8	273*	45·6	10·0	<8·5	82*	21·1	4·6	1·0	36*	17·7	2·1	0·7	34
	2	312	125·5	26·2	12·9	360*	54·1	11·1	14·4	74*	26·8	4·8	1·2	127*	16·6	2·1	0·6	31

Estimations of Br varied from 63 to 70%

The proportion of polyunsaturated acids present in the tumour was approximately of the same order as that present in the liver.

The results of Lavik & Baumann [1941] are interesting in this connexion. They found that the addition of fat to the basal diet of mice receiving applications of 20-methylcholanthrene to the skin increased tumour incidence from 12 to 83 % and that the tumour-promoting fraction was in the fatty acid fraction. They found, however, that dietary fat was much less effective in promoting induced skin tumours in rats though the rate of tumour formation was increased by oil applied locally. Their basal diet, however, already contained a considerable proportion of fat.

Our present results confirm those which we obtained when the fat-starved rats were fed with small doses of the essential unsaturated acids and seem to establish that when new tissue is formed in the rat, arachidonic, or some similar acid, is used up during the process of growth, being drawn especially from the subcutaneous fat.

In the fat-free animals it was previously found that when increase of weight followed the administration of small doses of arachidonic acid, the proportion of fat present in the tissues fell and it was suggested that the growing cell might directly utilize stored fat in some process in which the essential unsaturated acids also played a part.

In the above-described experiments where the animals were fed throughout with a diet rich in fat, the proportion of fat in the tissues was so variable that no conclusions could be drawn.

In Exp. III (Table 1) all tumours planted in the experimental rats failed to develop or regressed. No analyses were carried out on these animals, but when the results of the analyses of the three animals which had been taken as controls were examined, they showed an exceptionally low content of arachidonic acid. The average amount of bromide precipitate from the skin and carcass fat of one rat of this group averaged 50 mg. against 100–150 mg. of the similar precipitate in controls for the animals in which tumours had grown. This result suggested the investigation of tumour-implantation in fat-starved rats where a low level of arachidonic acid exists; the particulars of this investigation are described in a subsequent communication.

Summary

Walker tumour tissue was implanted in rats of 100–150 g. weight, fed on a normal diet containing fish meal and oil cake. Large tumours developed in 10–14 days.

The proportion of lipoid substance to fat-free dry weight was determined in skin, carcass and liver and compared with the corresponding values in the controls. The figures were very variable and no significant differences were established.

The highly unsaturated acids were estimated as bromide insoluble in cold benzene and their ratio to the fat-free dry weight determined in skin, liver and carcass.

In all the tumour-bearing animals, there was a marked fall in this ratio in the subcutaneous tissue when compared with the corresponding figures for the normal control rats. No such difference was detected in the carcass and liver fat.

In the only experiment in which tumours failed to develop the controls showed abnormally low percentage ratio of highly unsaturated acid to the fat-free dry weight of the tissue.

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